Reversible Immobilization of Glycoamylase by a Variety of Cu^{2+}-Chelated Membranes

Gözde Baydemir,[1] Ali Derazshamshir,[1] Müge Andaç,[1] Cenk Andaç,[2] Adil Denizli[1]

[1]*Department of Chemistry, Hacettepe University, Ankara, Turkey*
[2]*Department of Pharmacology, Dicle University, Diyarbakır, Turkey*

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ABSTRACT: Glycoamylase (AMG) is an γ-amylase enzyme which catalyzes the breakdown of large α(1,4)-linked malto-oligosaccharides to glucose. It is an extracellular enzyme and is excreted to the culture medium. In this study, AMG was immobilized on a variety of metal affinity membranes, which were prepared by chelating Cu^{2+} ions onto poly(hydroxyethyl methacrylate) (PHEMA) using *N*-methacryloyl-(L)-histidine methyl ester (MAH), *N*-methacryloyl-(L)-cysteine methyl ester (MAC), and *N*-methacryloyl-(L)-phenylalanine methyl ester (MAPA) as metal-chelating comonomers for reversible immobilization of AMG. The PHEMAH, PHEMAC, PHEMAPA membranes were synthesized by UV-initiated photo-polymerization and Cu^{2+} ions were chelated on the membrane surfaces. Cu^{2+}-chelated membranes were characterized by swelling tests, SEM, contact angle measurements, elemental analysis, and FTIR. AMG immobilization on the Cu^{2+}-chelated membranes was performed by using aqueous solutions of different amounts of AMG at different pH values and Cu^{2+} loadings. Durability tests concerning desorption of AMG and reusability of the Cu^{2+}-chelated membranes yielded acceptable results. It was computationally determined that AMG possesses four likely Cu^{2+}/Zn^{2+} binding sites, away from the catalytic site, to which the metal-chelated membranes can be efficiently used. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: affinity membranes; IMAC; AMG; enzyme activity

INTRODUCTION

Glycoamylase (AMG; EC 3.2.1.3) is a type of enzyme which catalyzes the breakdown of malto-oligosaccharides to glucose. AMG is an extracellular enzyme and is excreted to the culture medium.[1] The primary commercial application of AMG is the production of glucose syrups from starch. These syrups can be used for fermentation, production of crystalline glucose, or as a starting material for fructose syrups.[2] AMGs have also been applied to the brewing industry in the production of low calorie beer, where maltodextrins in the malted barley are hydrolyzed to simple sugars, which can then be completely fermented by brewer's yeast. AMG is second to proteases in worldwide distribution and sales among industrial enzymes.[3] In industrial conventional enzymatic reactions, a mixture of the substrate and the soluble enzyme is incubated. After completion of each batch of reaction, the amylases are inactivated. Naturally, the process would be more economical if the enzyme could be reused, for example by immobilization.[4]

Immobilized enzymes have been widely used in food, fine chemical, and pharmaceutical industries because they provide many advantages over free enzymes including repeated or continuous use, easy separation of the product from reaction media, easy recovery of the enzyme, and improvement in enzyme stability.[5–7] Reversible immobilization could provide the possibility of using such enzymes in an immobilized form and, in this way, having the advantages of the use of immobilized enzymes, saving time and cost.[8–10] A wide variety of methods have been employed in the immobilization of enzymes, such as adsorption, entrapment, crosslinking, and covalent attachment.[11,12] Among these immobilization techniques, adsorption is the most general, easiest to perform and oldest protocol of physical immobilization methods. Simplicity and reversibility are the most important advantages of this method. But a strong adsorption between the enzyme and support should be achieved in the reversible immobilization methodology in order to prevent enzyme desorption from immobilization supports. Noncovalent immobilization technique such as metal-chelated adsorption of the enzyme on an adsorbent can be a good option because it saves time and labor for simple operation and the supports can be reused after desorption of the inactivated enzyme, in this way, reduce the final price and generate fewer residues.[13–15]

Immobilized metal ion affinity chromatography (IMAC) has become a widespread analytical and

Correspondence to: A. Denizli (denizli@hacettepe.edu.tr).

preparative separation method for therapeutic proteins, peptides, nucleic acids, hormones, and enzymes.[16,17] IMAC introduces a new approach for selectively interacting materials on the basis of their affinities for chelated metal ions. The separation is based on the interaction of a Lewis acid (electron pair donor), i.e., a chelated metal ion, with an electron acceptor group on the surface of the protein.[18] Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indolyl group of tryptophan and the thiol group of cysteine. Co-operation between neighboring amino acid side chains and local conformations plays an important role in protein binding. Aromatic amino acids and the amino-terminus of the peptides also have some contributions.[19] The low cost of metals and the reuse of adsorbents for hundreds of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.[20] Immobilization of AMG has been studied using various supports, such as activated carbon,[21] polymeric support,[22] and inorganic materials.[23] Drawback of the enzyme immobilization includes activity decrease and diffusion limitations of the substrate, the intermediate, and the product. The surface reaction or the diffusion of substrate and product in the pore may be the rate-controlling step in the immobilized enzyme system.[24] If the pore size is higher or the particle size smaller, the effect of pore diffusion of the substrate and intermediates would be expected to decrease, and this would lead to results closer to those obtained with free enzyme.[21]

In this study, three different monomers; *N*-methacryloyl-(L)-histidine methyl ester (MAH), *N*-methacryloyl-(L)-cysteine methyl ester (MAC), *N*-methacryloyl-(L)-phenylalanine methyl ester (MAPA) were used as monomer ligand. These monomer ligands were selected due to their high flexibility and durability properties. As MAC, MAH, and MAPA are very small and reactive molecules they can be easily polymerized and because of that there are no leakage problem from the matrix during further studies. Beside these properties, these ligands provide lower material consumption and low costs. The poly(2-hydroxyethyl methacrylate-(*N*)-methacryloyl-(L)-histidine methyl ester) (PHEMAH), poly(2-hydroxyethyl methacrylate-(*N*)-methacryloyl-(L)-cysteine methyl ester) (PHEMAC), poly(2-hydroxyethyl methacrylate-(*N*)-methacryloyl-(L)-phenylalanine methyl ester) (PHEMAPA) membranes were synthesized by UV-initiated photopolymerization. The PHEMAH, PHEMAC, and PHEMAPA membranes were chelated to Cu^{2+} ions by addition of pHEMAH, PHEMAC, pHEMAPA membranes to an aqueous solution of Cu^{2+} ions. The Cu^{2+}-chelated membranes were characterized by swelling tests, SEM, elemental analysis, FTIR, and contact angle measurements. AMG adsorptions on the metal-chelated membranes were performed by using several aqueous solutions of AMG at different concentrations, pH, and Cu^{2+} loadings. Results for the desorption of AMG, reusability of the metal-chelated membranes, and the effects of pH, temperature, and time on the enzyme activity are extensively discussed in the following sections. The metal binding sites on AMG was predicted by computational means.

EXPERIMENTAL

Materials

Glucoamylase (AMG, exo-1,4-α-D-glucosidase, EC 3.2.1.3) from *Aspergillus niger* (94.5 U/mg) was obtained from Sigma (St. Louis, MO) and used as received. Hydroxyethyl methacrylate (HEMA) was purchased from Fluka A.G. (Buchs, Switzerland), which was filtered through an activated basic alumina column, distilled under reduced pressure and stored at 4°C. Azobisisobutyronitrile (AIBN) was obtained from Fluka (Switzerland) and re-crystallized in methanol. All other reagents, unless otherwise specified, were of analytical grade and used without further purification. Laboratory glassware was kept overnight in a 5% nitric acid solution. All glassware was rinsed with deionized water and dried in a dust-free environment before use. Buffer and sample solutions were pre-filtered through a 0.2-μm membrane (Sartorius, Gottingen, and Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Preparation of MAH/MAC/MAPA monomers

Details of the preparation and characterization of MAH, MAC, and MAPA monomers are reported elsewhere.[25–27] Briefly, L-histidine methylester (5.0 g)/L-cystein methylester (5.0 g)/L-phenylalanine methylester (5.0 g), and hydroquinone (0.2 g) were separately dissolved in 100 mL dichloromethane. The mixture was transferred into a 250-mL three-neck round-bottomed flask via a dropping funnel. After cooling down to 0°C, triethylamine (10 mL) was added and the mixture was stirred magnetically under nitrogen atmosphere. Six milliliters of methacryloyl chloride were placed into the dropping funnel, which was then introduced dropwise to the reaction mixture in 10 min. The reaction chamber was then removed from the water bath and the reaction was maintained at room temperature for 2 h. Unreacted methacryloyl chloride was removed from the medium by extraction with 10% NaOH solution. The aqueous phase was

evaporated in a rotary evaporator. The reaction products MAH, MAC, and MAPA were stored as solutions of ethanol. The reaction scheme of ligand monomers was given in Figure 1.

Preparation of PHEMAH, PHEMAC, and PHEMAPA membranes

The PHEMAH, PHEMAC, PHEMAPA membranes were prepared by UV-initiated photo polymerization. Details of the preparation of PHEMA-based membranes are reported elsewhere.[27] The membrane preparation mixture (5.0 mL) contained 2.0 mL of HEMA and 50 mg of the MAH, MAC, or MAPA monomer, respectively, for each of the polymers, 20 mg of AIBN as the polymerization initiator and 3.0 mL of Tris-HCl buffer (50 m*M*, pH 7.0). The resulting mixtures were equilibrated at 25°C for 30 min in a water bath, which were then poured into the molds and exposed to long-wave ultraviolet radiation for 1.0 h. The membranes were washed several times with distilled water, ethanol (70%, v/v), and Tris-HCl buffer in a sonicated water bath and were cut into circular pieces (diameter: 0.5 cm) with a perforator.

Incorporation of Cu^{2+} ions

The chelation of Cu^{2+} ions was performed in batch system. The chelation of Cu^{2+} ions to PHEMAH, PHEMAC, and PHEMAPA membranes was implemented as follows: 100 mg of each of the membrane pieces were separately mixed with 100 mL of aqueous solutions containing 50 ppm Cu^{2+} ions, whose pH was adjusted at 5.0 using 0.1*M* HCl and 0.1*M* NaOH stock solutions, to provide optimal conditions for Cu^{2+} chelate formation at room temperature. An aqueous solution containing 10% HNO_3 and 1000 ppm Cu^{2+} was used as a standard for atomic absorption studies. The flasks containing the Cu^{2+}-chelated membrane solutions were stirred magnetically at 100 rpm for 1 h. The concentration of the Cu^{2+} ions in the resulting membrane solutions was determined by a graphite furnace atomic absorption spectrometer (AAS 5EA, Carl Zeiss Technology, Zeiss Analytical Systems, Germany). These experimental determinations were implemented for each of the Cu^{2+}-chelated membrane solutions to determine the mean values and standard deviations by standard statistical methods. Confidence intervals of 95% were calculated for each set of samples in order to determine the margin of error. The amount of Cu^{2+} ions loaded on membranes was determined by taking the difference between the initial and equilibrium metal ion concentrations in solutions. The Cu^{2+} ion leakages of the PHEMAH, PHEMAC, and PHEMAPA membranes were investigated by suspending each membrane in media at pH 7.0–11.5, which were equilibrated by stirring for 24 h at room temperature. The Cu^{2+} ion concentrations in the supernatants were determined by AAS. The metal-chelated membranes were stored at 4°C in the presence of 0.02% sodium azide to prevent microbial contamination.

Characterization of Cu^{2+}-chelated membranes

The extent to which MAH, MAC, and MAPA incorporated in the PHEMAH, PHEMAC, and PHEMAPA membranes was determined by measuring the C, H, N, O contents by a LECO (Model CHNS-932) elemental analyzer. The dry PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes were coated with gold under reduced pressure and their scanning electron micrographs were obtained by a JEOL (JSM 5600, Japan) scanning electron microscope. Water uptake ratios of pHEMAH-Cu^{2+}, pHEMAC-Cu^{2+}, and pHEMAPA-Cu^{2+} were determined in distilled water as follows: initially dry membranes were carefully weighed out and placed into 50 mL vials containing a medium of water uptake. Each vial was placed into an isothermal water bath at 25°C for 24 h. The polymer samples were taken out of the media, wiped out with a filter paper, and weighed out. The weight ratios of the dry and wet samples were recorded. The water uptake ratio was calculated using the following expression:

$$\text{Swelling ratio}(\%) = [(W_s - W_o)/W_o] \times 100 \quad (1)$$

where W_o is the weight of dry membrane and W_s is the weight of wet membrane.

Wettability studies on membranes were performed by the captive bubble method. The membrane samples were initially wetted with water, which were then placed in an environmental chamber to obtain contact angles. Three test liquids including formamide, toluene, and heptane were separately used to generate a bubble on the surface of floating wet membranes facing down in water, which was used for contact angle determination by video image evaluation on a DSA 100 contact angle meter, KRUSS (Germany). Fifteen determinations for each membrane sample were obtained to average out the contact angles. The liquid contact angle is a product of the difference between the average contact angle and 180°, a value determined automatically by the DSA 100 instrument. In addition, right and left contact angles as well as drop dimension parameters were computed automatically by the DSA100 instrument. Surface free energies of the membranes were computed and compared by utilizing the contact angles in Fowkes approach.[28]

The wetting of a solid surface by a liquid and the concept of contact angle (θ) were first formulated by the Young equation [eq. (2)][29];

$$\gamma_l \cos\theta = \gamma_s - \gamma_{sl} \quad (2)$$

where γ_l is the surface energy of the liquid (mN/m), γ_{sl} is the interfacial energy of solid/liquid interface (mN/m), and γ_s is the surface energy of solid (mN/m).

By using the Fowkes approach, polar and disperse fractions of the surface free energy of a solid can be obtained by utilizing a geometric mean approach, which yields the following equation when combined with Young's equation [eq. (2)]:

$$\gamma_l(1+\cos\theta) = 2[(\gamma_l^p\gamma_s^p)^{1/2} + (\gamma_l^d\gamma_s^d)^{1/2}] \quad (3)$$

Equation (3) can be rearranged as eq. (4) as proposed by Owens and Wendt coworkers:

$$\gamma_l(1+\cos\theta)/(\gamma_l^d)^{1/2} = [(\gamma_s^p)^{1/2}(\gamma_l^p)^{1/2}/(\gamma_l^d)^{1/2}] + (\gamma_s^d)^{1/2} \quad (4)$$

where θ is the contact angle, γ_l is the liquid surface tension (mN/m) and γ_s is the solid surface tension or free energy (mN/m). The superscripts d and p refer to the dispersive and polar components, respectively. Equation (4) is a linear equation as in $y = mx + b$, where x equals to $(\gamma_l^p)^{1/2}/(\gamma_l^d)^{1/2}$ and can be plotted versus y that is γ_l $(1+\cos\theta)/(1+\gamma_l^d)^{1/2}$. The slope of such a linear plot is $(\gamma_s^p)^{1/2}$ and the y-intercept is $(\gamma_s^d)^{1/2}$. The total free surface energy is merely the sum of its two component forces.

Computational studies

Refinement of Zn^{2+} metal ions, as a substitute of Cu^{2+}, in complex with AMG was implemented by AMBER v11 (2010) suite of programs.[30] Crystal structure of AMG from *Aspergillus niger* was obtained from the Protein Data Bank, PDB ID: 3EQA. Pang's[31] tetrahedron-shaped Zn^{2+} model was applied during the AMBER treatment of the protein–metal ion complex. Each tetra-coordinated Zn^{2+} metal ions included two covalently linked hydroxyl groups. Three cystine bonds, CYS(:234)—CYS(:237), CYS(:246)—CYS(:473), and CYS(:286)—CYS(:294), were defined in the AMG protein. The parm99SBildn[32] force field parameters for the AMG protein and Pang's tetrahedron-shaped Zn force field parameters[31] were applied for a short minimization of the protein–Zn complex. Initially, the protein and the OH^- ions bound to the Zn^{2+} ions were held fixed using a 500 kcal/mol Å^2 force constant while the Zn^{2+} ions were allowed to relax over 100 iterations. Secondly, only the protein coordinates were restrained using a 500 kcal/mol Å^2 force constant while the Zn^{2+} ions and the hydroxyl coordinates were relaxed for 500 iterations. Finally, all the restrains were removed and the entire protein–Zn complex was relaxed for another 1000 iterations.

Immobilization of AMG

To evaluate the effect of ligand density on the interactions between the metal ion and the target protein, AMG immobilization studies were performed using various amounts of Cu^{2+}-chelated membranes. Briefly, 0.5 mg/mL AMG was prepared, followed by exposure to 100 mg of Cu^{2+}-chelated (in the range of 10–100 ppm) PHEMA, PHEMAH, PHEMAC, and PHEMAPA membranes. AMG immobilization on the Cu^{2+}-chelated membranes was tested at various pH values, including sodium acetate buffer (0.1*M*, pH 3.5–5.5) and phosphate buffer (0.1*M*, pH 6.0). The Cu^{2+}-chelated membranes were added to 20 mL of AMG solutions (1.0 mg/mL) prepared using the defined buffer solutions. The resulting suspensions were subsequently incubated at 25°C by shaking at 100 rpm until adsorption equilibrium was reached. The AMG-immobilized Cu^{2+}-chelated membranes were separated from the enzyme solution via decantation, which were then rinsed with the same buffer solution three times. The elution solutions containing residual AMG were collected. The activities of immobilized AMG were tested by the relative activity assay.[33] The resulting AMG immobilized membranes were stored at 4°C in a fresh buffer solution until use. The amount of the protein in the enzyme solution and in the washing solution was determined by the Bradford method. To determine the adsorption capacity of the Cu^{2+}-chelated membranes, various concentrations of AMG, ranging from 0.1 mg/mL to 2.5 mg/mL, were prepared and used for immobilization. A calibration curve was constructed using various concentrations of AMG solutions, 0.05–3.00 mg/mL. The amount of AMG adsorbed onto the membranes was determined by the Bradford method by subtracting the visible absorbance (λ = 595 nm) before and after the adsorption, for which the measurements were implemented using a UV–vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601).

Effects of pH, temperature, and time on free and immobilized AMG activities

Activities of free and immobilized AMG were tested under various conditions by changing pH or temperature or time. Briefly, 0.5 mg of free/immobilized AMG was dissolved in 0.5 mL of 0.1*M* acetate buffer solution (pH 4.5), to which 1.0 mL of soluble starch solution containing 1.0% (w/v) soluble starch, which was gelatinized in water by continuously stirring for 15 min at 100°C, was added as the substrate of AMG. After enzyme–substrate interaction, 10 mL of NaOH solution (0.1*M*) was added at 60°C to end the enzyme reaction. The amount of glucose released into the enzyme reaction medium was determined

by the conventional dinitrosalicylic acid (DNS) method. Reversibly immobilized AMG activities were measured out of three determinations, for which the relative standard deviations were found to be less than 3.0%. One unit of enzyme activity (U) is given as the amount of enzyme that produces 1.0 μmol of glucose from soluble starch per minute under a defined condition. The relative activity of the free/immobilized enzyme is calculated by the following equation:

$$R(\%) = (A_{\text{im}}/A_{\text{free}}) \times 100 \quad (5)$$

where R is the relative activity of the immobilized enzyme (%), A_{im} is the activity (U) of the immobilized enzyme, and A_{free} is the activity of the same amount of free AMG in solution as that immobilized on membranes.

Desorption and reusability of the membranes

In order to determine the reusability of membranes, AMG adsorption and desorption cycles were repeated 10 times. AMG desorption of the PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes were carried out using a 1.0*M* NaCl solution at pH 8.0. The membranes were then rinsed several times with an acetate buffer solution (50 m*M*, pH 4.0), which were then reused in the enzyme re-immobilization. The specific activity of immobilized AMG on various membranes was defined at optimal conditions for each membrane. Specific activity of AMG defined as number of micromoles of glucose which was liberated in 1 min by 1 mg of dried immobilizate at 55°C and for 3 min.

RESULT AND DISCUSSION

Characterization of membranes

The chemical content of the MAH, MAC, and MAPA incorporated into the PHEMAH, PHEMAC, PHEMAPA were determined based on nitrogen stoichiometry by elemental analysis, which were found to be 96 μmol, 102 μmol, and 78 μmol, respectively, per gram of the polymer.

SEM photographs showing the surface structure of the Cu^{2+}-chelated PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes are presented in Figure 2(A–C), respectively. As seen in the figures, the Cu^{2+}-chelated membranes possess highly porous surfaces, suitable for protein docking. The high porosity of the surface structures is the major factor to increase surface area for more efficient protein–metal interactions. In addition, these large pores reduce the mass transfer resistance and facilitate the diffusion of proteins due to low diffusional resistance in the membrane, implying high adsorption capacity and rate.[27]

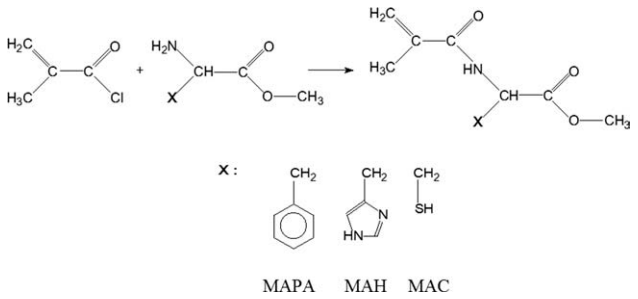

Figure 1 Reaction scheme of ligand monomers.

The PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes are hydrophilic structures. They rather swell in aqueous solutions but do not dissolve. Initial adsorption of water weakens the secondary bonds within the membranes, which widens the distance between the polymer chains, ultimately enabling water uptake. The water swelling ratios of the PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes were found to be 123.2, 110.9, and 90.2%, respectively, which are quite sizable as compared to that of PHEMA (62.4%) (Table I). It is highly likely that incorporation of a monomer group such as MAH, MAC, and MAPA into PHEMA increases hydrophilicity.[27] As MAPA is more hydrophobic than MAC and MAH, PHEMAPA-Cu^{2+} has the least swelling ratio (90.2%) in comparison to the PHEMAC-Cu^{2+} and PHEMAH-Cu^{2+} membranes.

Characterization of the surface chemistry is a crucial issue in affinity membranes. The contact angle technique is used routinely in the characterization of biomaterials to describe hydrophilicity or to estimate surface free energy. Please note that the contact angle values in Table II were given are not the difference between the average contact angle and 180° so a relatively small contact angle indicates a relatively more hydrophilic surface. It is worth noting that contact angles are measured through the water phase, so this value was interpreted conversely to that of usual wettability numbers, e.g., the higher the contact angle this number is the greater wetting by the liquid contained in the captive bubble cuvette.[27] The equilibrium contact angles were given in Table II based on the captive bubble method.

As seen in Table II, the most hydrophilic surface belongs to the PHEMA membrane, e.g., the greater the contact angle is the greater the hydrophilicity. Incorporation of the MAH, MAPA, and MAC functional monomers into the PHEMA polymer decreases slightly the hydrophilicity of the resulting membrane. In contrast, the chelation of Cu^{2+} ions onto membrane surfaces seems to slightly increase

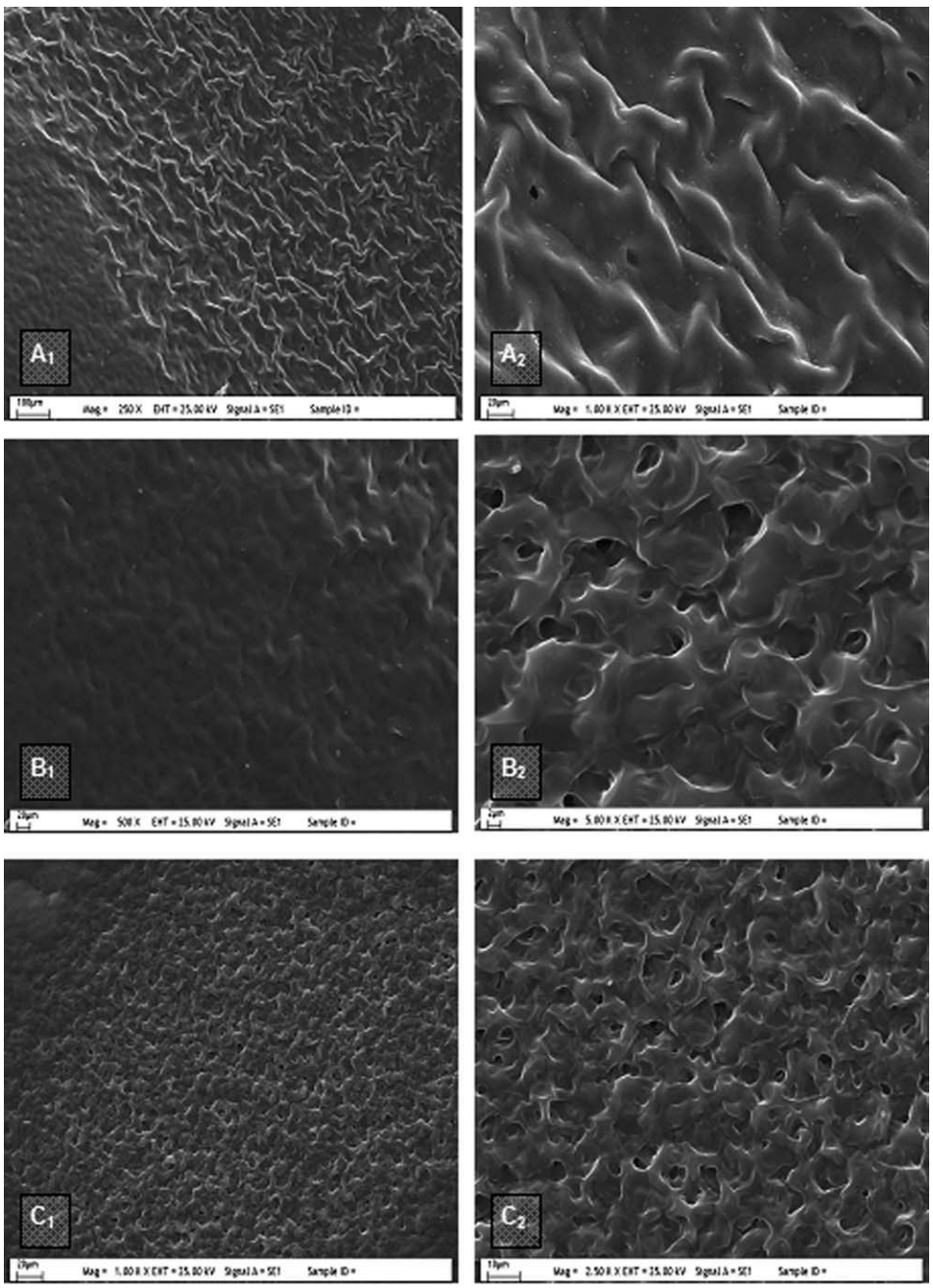

Figure 2 SEM images of the PHEMAH-Cu^{2+} (A_1 and A_2); PHEMAC-Cu^{2+} (B_1 and B_2), and PHEMAPA-Cu^{2+} (C_1 and C_2) membranes.

contact angles, so does the hydrophilicity of the metal-chelated membranes. It was observed that the contact angles decreased significantly after reversible immobilization of AMG onto the Cu^{2+}-chelated membranes PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+}. Furthermore, the reversible immobilization capacity of the PHEMAH-Cu^{+2} membrane for the AMG was higher than those of PHEMAC-Cu^{2+} and PHEMAPA-Cu^{2+}. These results suggest that the AMG adsorption depends on the structure

TABLE I
% Swelling Ratio of the Membranes

	PHEMA	PHEMAH	PHEMAC	PHEMAPA	PHEMAH-Cu^{2+}	PHEMAC-Cu^{2+}	PHEMAPA-Cu^{2+}
Swelling ratio %	62.4	105.6	112.2	87.7	110.9	123.2	90.2

TABLE II
Contact Angles of Membranes in Liquids: Toluene, Heptane, and Formamide

Membrane samples	Liquids		
	Toluene ($\gamma_l = 28.4$) CA (θ°)	Heptane ($\gamma_l = 20.1$) CA (θ°)	Formamide ($\gamma_l = 58.0$) CA (θ°)
PHEMA	64.1	57.5	148.0
PHEMAH	53.2	49.1	146.0
PHEMAPA	47.0	38.3	133.0
PHEMAC	54.6	41.8	144.0
PHEMAH-Cu^{2+}	45.6	43.3	135.0
PHEMAPA-Cu^{2+}	36.0	30.5	121.0
PHEMAC-Cu^{2+}	51.2	39.1	142.9
PHEMAH-Cu^{2+}-AMG	39.5	37.1	128.2
PHEMAPA-Cu^{2+}-AMG	29.3	27.3	113.1
PHEMAC-Cu^{2+}-AMG	47.4	22.4	137.6

γ_l = surface free tension.

and distribution of functional groups on the surface of the PHEMA membrane (Fig. 2).

Total surface energies and their dispersive and polar components, computed by using the contact angles in Fowkes method for wet membranes studied in our laboratory are presented in Table III. As seen in the table the polar component make the major contribution to the total surface free energy in virtually all the membranes tested, excluding pHEMAPA-Cu^{2+}-AMG, as polar groups on the membrane surface make favorable contacts with surface water molecules.

It is observed in Table III that the total surface free energies (γ) of the membranes and their polar energy components decrease at variable extends as Cu^{2+} and AMG are immobilized sequentially on the membranes, a finding associated with a decrease in the hydrophilicity and hydroxyl density of membrane surfaces.[34] The total surface free energy as well as its polar component of PHEMAH seems to be the most affected membrane species (Table III) by Cu^{2+} chelation, which possibly deprotonate imidazoline group of histidine ultimately diminishing its polarity. It was determined that AMG adsorption significantly changed the relatively more hydrophobic nature of the PHEMAPA-Cu^{2+} surface (γ = 33.1 mN/m), making it more hydrophilic after adsorption (γ = 38.7 mN/m). AMG adsorption on the PHEMAH-Cu^{2+} and PHEMAC-Cu^{2+} membranes, however, did not make such drastic changes as these membranes are already hydrophilic. Because the PHEMAH-Cu^{2+} membrane has the highest hydrophilic nature with a total surface energy (γ) of 44.4 mN/m and a polar energy (γ^p) of 25.3 mN/m, and it possesses the greatest tendency to adsorb, it is highly likely that hydrophilic interactions are the predominant forces driving the AMG adsorption.

Considering the energy values given in Table III, it is concluded that the surface Cu^{2+} ions noticeably enhances AMG adsorption and the adsorption strongly depends on the dispersive and polar energy components of the total surface free energies for the Cu^{2+}-chelated membranes.

Effect of Cu^{2+} concentration on Cu^{2+} loading

The amount of Cu^{2+} chelated on the PHEMAH, PHEMAC, and PHEMAPA membranes, treated with adsorption media containing Cu^{2+} ions, was measured by AAS, whose results are reported in Table IV. As seen in table, the maximum amount of Cu^{2+} incorporation is observed with the PHEMAH-Cu^{2+} membrane due possibly to three likely high-affinity coordination from MAH to Cu^{2+} ion. Based on the characterization results reported in "Characterization of membranes" involving the elemental analysis, nitrogen stoichiometry of the monomers MAH, MAPA, and MAC incorporated into the corresponding polymer membranes, and the AAS results, those obtained with 100 mg/L Cu^{2+} adsorption media as shown Table IV, it is approximately determined that the ratio of μmoles of monomer : μmoles of bound Cu^{2+} ion per a gram of the corresponding polymer is 96 : 65 (roughly 1 : 1) for PHEMAH-Cu^{2+}, 78 : 42 (roughly 2 : 1) for PHEMAPA-Cu^{2+}, and 102 : 38 (roughly 3 : 1) for PHEMAC-Cu^{2+}. The AAS measurements showed that there was no detectable leakage of Cu^{2+} from membranes in any of the adsorption and desorption media.

Reversible immobilization of AMG

Effect of Cu^{2+} loading amount

Chelated-Cu^{2+} amount plays a crucial role inn the interaction between the Cu^{2+}-chelated ligand and AMG, the target protein, whose relationship is graphically shown in Figure 3 for the PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes. The *x*-axis in the figure represents the concentration of

TABLE III
Total Surface Energies (γ) and their Dispersive (γ^d) and Polar (γ^p) Components Determined by Fowkes Approach

Membrane samples	γ (mN/m)	γ^d (mN/m)	γ^p (mN/m)
PHEMA	47.2	14.6	32.5
PHEMAH	55.1	20.8	34.1
PHEMAPA	33.3	17.1	16.2
PHEMAC	34.8	15.5	19.2
PHEMAH-Cu^{2+}	44.4	19.1	25.3
PHEMAPA-Cu^{2+}	29.9	13.8	16.0
PHEMAC-Cu^{2+}	33.1	15.4	17.7
PHEMAH-Cu^{2+}-AMG	41.7	21.2	20.5
PHEMAPA-Cu^{2+}-AMG	38.7	22.8	15.8
PHEMAC-Cu^{2+}-AMG	32.4	12.9	19.4

TABLE IV
Amount of Chelated Cu^{2+} Ions on Membranes as Determined by AAS

Initial Cu^{2+} concentration in media used (mg/L)	Initial Cu^{2+} concentration in media used (μmol/L)	Bound Cu^{2+} on PHEMAH (μmol Cu^{2+}/96 μmol MAH/g membrane)	Bound Cu^{2+} on PHEMAPA (μmol Cu^{2+}/78 μmol MAPA/g membrane)	Bound Cu^{2+} on PHEMAC (μmol Cu^{2+}/102 μmol MAC/g membrane)
10	157	38.0	21.0	16.2
20	315	47.0	29.0	22.0
30	473	53.2	32.6	31.5
50	788	64.6	42.0	33.2
75	1182	64.1	43.0	37.8
100	1577	64.8	42.5	38.3

Cu^{2+} media used to load Cu^{2+} onto metal-free membranes while the *y*-axis is the amount of AMG reversibly immobilized by adsorption on the Cu^{2+}-chelated membranes. As seen in the figure, the AMG immobilization increases logarithmically up to about 50 mg/L of Cu^{2+} media used, beyond which the AMG immobilization saturates at increased Cu^{2+} media concentrations. This finding strongly suggests that AMG possesses one or more Cu^{2+} binding sites and it requires an optimal Cu^{2+} surface density on the membranes to appropriately bind AMG without steric hindrance effects.

According to the Lewis acid–Lewis base concept of Pearson, Cu^{2+} ion, as a soft or borderline Lewis acid, is more likely to accept non-bonding lone pair electrons from amino nitrogen atoms of heterocyclic or aliphatic groups, in particular from the imidazole group of the histidine residues in proteins.[35] This notion is supported by the AMG adsorption plots in Figure 3, showing that the PHEMAH-Cu^{2+} membrane immobilized more AMG than the PHEMAC-Cu^{2+} and PHEMAPA-Cu^{2+} membranes.

The initial concentration of AMG solution used is 0.5 mg/mL, the total volume of Cu^{2+}-chelated polymer and AMG solution (V_{tot}) is 10 mL and the dry mass of Cu^{2+}-chelated polymer ($m_{dry\ membrane}$) is 0.044 g for PHEMAH-Cu^{2+}, 0.039 g for PHEMAC-Cu^{2+}, and 0.048 g for PHEMAPA-Cu^{2+}. The experiments were carried out for 2 h at 25°C.

Effect of pH

In general, the maximum adsorption of proteins by solid supports is observed at pHs around the isoelectric point (pI) with no net protein charges.[36] The pI of AMG is 4.5. As seen in Figure 4, in which enzyme adsorption is plotted versus pH, AMG is maximally adsorbed at pH 4.0 for PHEMAH-Cu^{2+}, at pH 5.0 for PHEMAC-Cu^{2+}, and at pH 6.0 for PHEMAPA-Cu^{2+}. As AMG possesses a constant pI it then becomes highly likely that different optimal-pH values obtained with AMG bound to different membranes predominantly depend on the optimal pH to appropriately coordinate Cu^{2+} on the corresponding membrane surface. Optimal pH conditions would possibly provide suitable electrostatic and/or coordination interactions between ionizable amino acid side chains of AMG and chelated-Cu^{2+} ions The amount of adsorbed AMG drastically decreases at pH values lower and higher than the optimum pHs due possibly to different ionization and conformational states of AMG. Therefore, all other AMG adsorption experiments were carried out at the aforementioned optimal pH values.

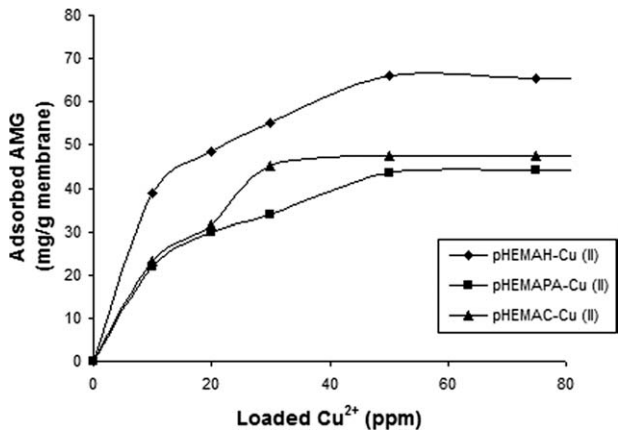

Figure 3 The effect of Cu^{2+} chelation amount on AMG adsorption.

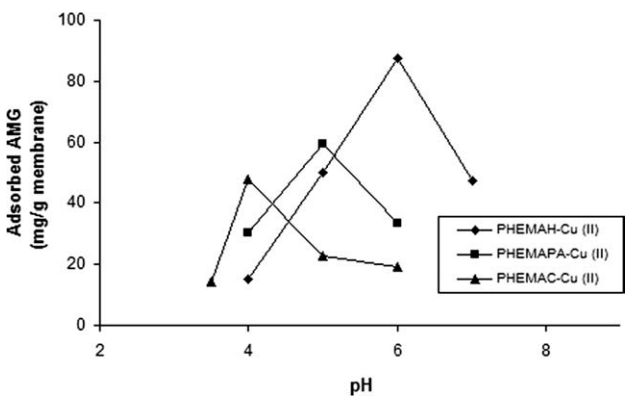

Figure 4 The effect of pH on AMG immobilization. Initial concentration of AMG: 0.5 mg/mL, *T*: 25°C.

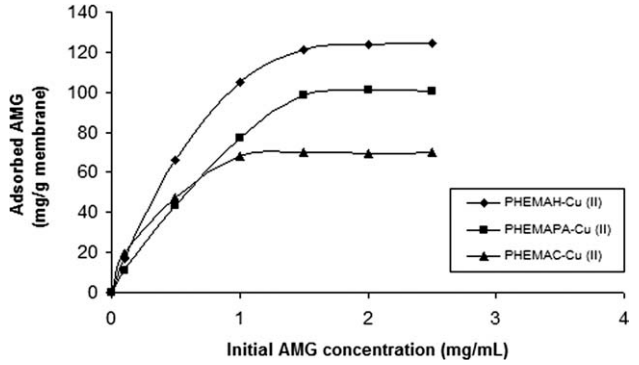

Figure 5 The effect of initial AMG concentration on AMG immobilization. pH 6.0; *T*: 25°C.

Effect of AMG concentration

Figure 5 shows the effect of initial AMG concentration on reversible AMG immobilization. As seen in the figure the AMG adsorption on Cu^{2+}-chelated membranes increases as the initial AMG concentration in adsorption media is increased, which ultimately saturates above the initial AMG concentration of 1.5 mg/mL. The amount of AMG immobilized on the Cu^{2+}-chelated membranes was found to be 121.2 mg/g for PHEMAH-Cu^{2+}, 98.4 mg/g for PHEMAPA-Cu^{2+}, and 69.0 mg/g for PHEMAC-Cu^{2+}. The amount of AMG immobilization on metal-free membranes was 12.8 mg/g for PHEMAH, 4.8 mg/g for PHEMAC, and 6.6 mg/g for PHEMAPA. Apparently, the Cu^{2+}-chelation of the membranes significantly increased AMG adsorption due most likely to specific interactions between the chelated-Cu^{2+} ions and the surface amino acid residues of AMG.

Reversibly immobilized AMG activity

Effect of pH

The effect of pH on the activity of free/immobilized AMG was studied in the range of pH 3.0–7.0. As shown in Figure 6 the relative activity of unbound

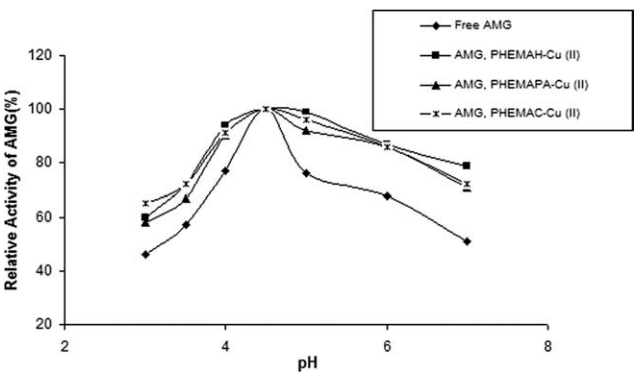

Figure 6 The effect of pH on the activity of free/immobilized AMG.

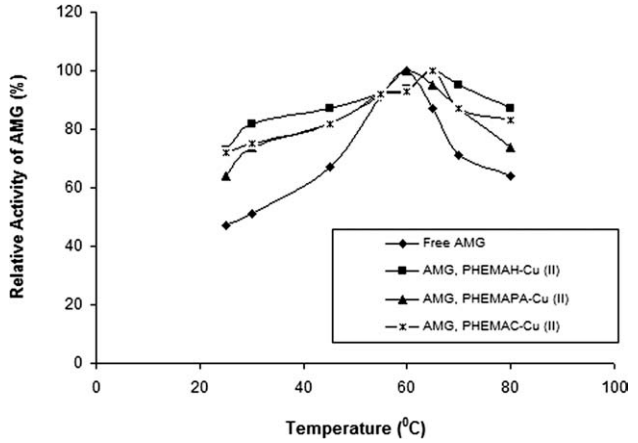

Figure 7 The effect of temperature on the activity of free/immobilized AMG.

AMG peaks at pH 4.5, which corresponds to the pI of AMG. The activity of unbound AMG decreases drastically at pH values lower and higher than pI. Furthermore, the relative activity of AMG immobilized on the PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes as observed in Figure 5 slightly increase below and above the pI point as compared to unbound AMG, suggesting that the immobilization process increases the AMG stability and the enzyme activity becomes more pH-independent.

Effect of temperature

The optimal temperatures that free AMG functions are 60°C as seen in Figure 7. The free enzyme activity abruptly decreases above and below the optimal temperature. Interestingly, it was found that immobilized AMG is relatively able to sustain its activity above and below the optimal temperature, strongly suggesting that immobilization increases the enzyme durability on temperature changes.[37]

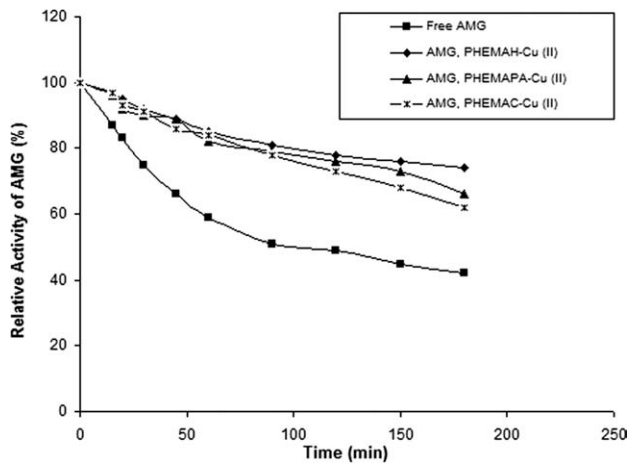

Figure 8 Effect of time on the activity of free/immobilized AMG.

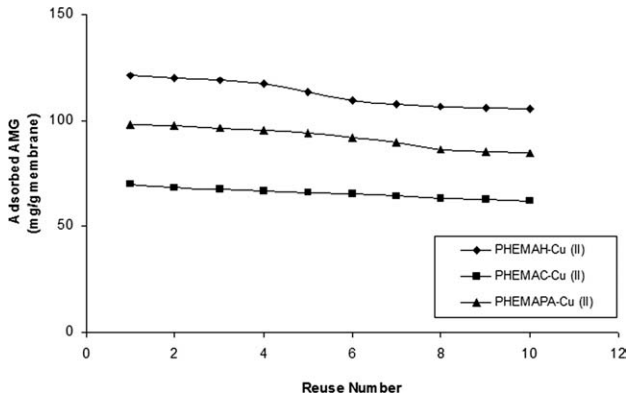

Figure 9 Reusability plots of Cu^{2+}-chelated membranes. pH 6.0; *T*: 25°C; desorption time:1 h.

Storage stability

In order to determine the time effect on AMG activity in acetate buffer at 60°C, free AMG and AMG immobilized on the Cu^{2+}-chelated pHEMAH-Cu^{2+}, pHEMAC-Cu^{2+}, and pHEMAPA-Cu^{2+} membranes were incubated in acetate buffer at 60°C for 180 min during which the enzyme activities were recorded at varying time intervals. The activity results plotted versus time, shown in Figure 8, indicate that the storage stability of immobilized AMG was higher than that of free AMG, suggesting that the immobilization of AMG onto Cu^{2+}-chelated membranes increases the storage stability of AMG as compared to free AMG.[38] Finally it was determined that the storage stability of immobilized AMG improves greatly at 4°C.

Desorption and reusability

Desorption experiments were performed for all AMG-immobilized membranes by using a 1*M* NaCl solution at pH 8.0. The AMG-desorbed Cu^{2+}-chelated membranes were then re-subjected to adsorption and desorption tests for a total of 10 times. It was determined that adsorbed AMG was successfully desorbed from the PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes with desorption ratio of 87%, 89%, and 86%, respectively. As seen in Figure 9, no significant decrease in the adsorption capacities of the membranes were observed after 10 cycles of adsorption–desorption tests. As reported in Table V specific activities of AMG desorbed from the PHEMAH-Cu^{2+}, PHEMAPA-Cu^{2+}, and PHEMAC-Cu^{2+} membranes were found to be very high, 98.3 U/mg, 95.3 U/mg, and 97.1 U/mg, respectively, a finding which supports reversible immobilization of AMG and long-term reusability of the Cu^{2+}-chelated membranes.

TABLE V
Specific Activity of AMG

	Free AMG[a]	PHEMAH-Cu^{2+}-AMG[a]	PHEMAPA-Cu^{2+}-AMG[a]	PHEMAC-Cu^{2+}-AMG[a]
Specific activity (u/mg)	94.5	98.3	95.3	97.1

[a] Sample volume: 10 mL, protein content: 2 mg/mL.

Cu^{2+}/Zn^{2+} binding sites on AMG

The X-ray structure of AMG from *Aspergillus niger*, deposited at the Protein Data Bank by J. Lee and M. Paetzel (to be published), PDB Code: 3EQA, contains amino acid residues 25 through 494. In similar spirit to the X-ray structure of an AMG from

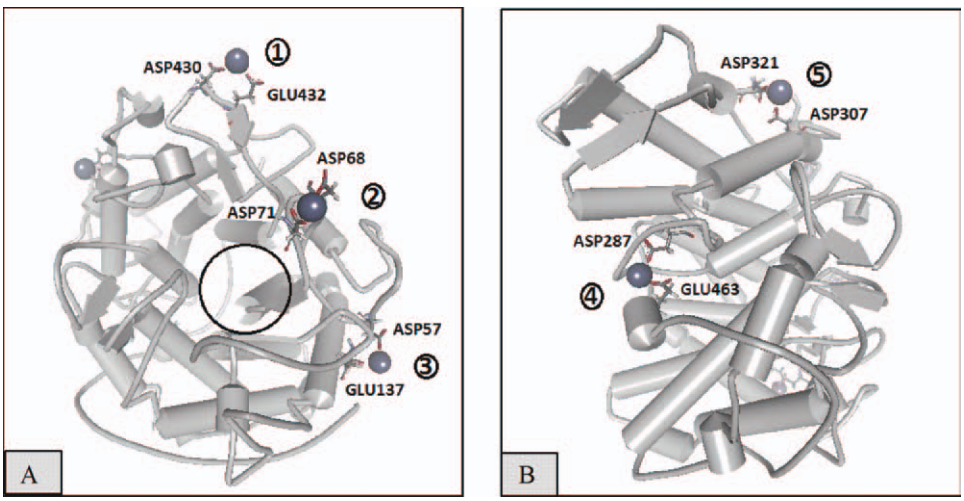

Figure 10 Illustration of the most likely Cu^{2+} binding sites on the surface of the AMG from *Aspergillus niger*, PDB Code: 3EQA. A: A front view of the protein–metal complex, on which three Zn^{2+}/Cu^{2+} binding sites are annotated, and (B) a back view of the complex, on which two Zn^{2+}/Cu^{2+} binding sites are annotated. The enzyme catalytic site is circled in (A). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hyperthermophilic archaeon *Pyrococcus woesei* in complex with nine surface Zn^{2+} metal ions,[39] PDB Code: 1MXD, the most likely Zn^{2+}/Cu^{2+} metal ion binding sites on the AMG from *Aspergillus niger*, PDB Code: 3EQA, were determined by protein surface scanning for aspartate, glutamate, histidine, cystein, and lysine aminoacid residues. As a result, five likely metal binding sites were located on the enzyme surface based on the proximity between the carboxylate groups of certain aspartate and glutamate residues. The Cu^{2+}/Zn^{2+} binding sites found on the from *Aspergillus niger* are shown and annotated in Figures 10(B) (back view) and 10(A) (front view), which are numbered **1** for ASP(:430)-(Zn/Cu)$(OH)_2$-GLU(:432), **2** for ASP(:68)-(Zn/Cu)$(OH)_2$-ASP(:71), **3** for ASP(:57)-(Zn/Cu)$(OH)_2$-GLU(:137), **4** for ASP(:287)-(Zn/Cu) $(OH)_2$-GLU(:463), and **5** for ASP(:307)-(Zn/Cu)$(OH)_2$-ASP(:321). The Cu^{2+}/Zn^{2+} binding site **2**, Figure 10(A), is located in the vicinity of the enzyme catalytic site, circled in Figure 10(A), which would significantly lower/disable the activity of AMG in the catalytic core if the metal-chelated membranes were bound to this metal binding site. Therefore, the remaining metal binding sites **1** and **3**, Figure 10(A), and **4** and **5**, Figure 10(B), are more likely to bind the metal-chelated membranes without diminishing the catalytic activity.

CONCLUSION

Affinity membranes with specifically functionalized surfaces play a crucial role in reversible immobilization of proteins.[40] The surface properties, the chemical, biological, and mechanical stabilities, and the functional ligand density of the membrane materials can be improved during preparation steps of membranes. This study describes the preparation of Cu^{2+}-chelated PHEMA membranes carrying MAH (PHEMAH), MAC (PHEMAC), and MAPA (PHEMAPA) and their applications in affinity chromatography for reversible immobilization of AMG. The PHEMA, PHEMAH, PHEMAC, and PHEMAPA membranes were prepared by UV-initiated photo polymerization. This followed the immobilization of Cu^{2+} ions on the membranes. The PHEMAH-Cu^{2+}, PHEMAC-Cu^{2+}, and PHEMAPA-Cu^{2+} membranes were found to exhibit high adsorption capacity for AMG. This finding was supported by computational studies showing that AMG possesses four likely high-affinity binding sites for Cu^{2+}. It was determined that the activities of immobilized AMG retained over wider temperature and pH ranges than that of free AMG. Comparison of the total surface free energies of the membranes with their polar energy components revealed that the PHEMAH-Cu^{2+} membranes possess the highest hydrophilic surface density which leads to the greatest AMG immobilization capacity. It was determined that reversibly immobilized AMG as well as the Cu^{2+}-chelated membranes are reusable without significant AMG activity decrease. This indicates that immobilized AMG is a suitable probe to be used in a continuous system for large-scale production of glucose in industry.

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References

1. Na, B. G.; Yang, C. Korean Biochem J 1985, 18, 222.
2. Fogarty, W. M. Microbial Enzyme and Biotechnology; Applied Science Publisher: London, 1983.
3. James, J. A.; Lee, B. H. J Food Biochem 1997, 21, 1.
4. Akgöl, S.; Denizli, A. J Mol Catal B 2004, 28, 7.
5. Özturk, N.; Akgöl, S.; Arısoy, M.; Denizli, A. Sep Purif Technol 2007, 58, 83.
6. Akgol, S.; Ozturk, N.; Karagozler, A. A.; Uygun, D. A.; Uygun, M.; Denizli, A. J Mol Catal B 2008, 51, 36.
7. Özturk, N.; Tabak, A.; Akgöl, S.; Denizli, A. Colloid Surf A 2008, 322, 148.
8. Kara, A.; Osman, B.; Yavuz, H.; Besirli, N.; Denizli, A. React Funct Polym 2005, 62, 61.
9. Phadtare, S.; D'Britto, V.; Pundle, A.; Prabhune, A.; Sastry, M. Biotechnol Prog 2004, 20, 156.
10. Osman, B.; Kara, A.; Uzun, L.; Besirli, N.; Denizli, A. J Mol Catal B 2005, 37, 88.
11. Marín-Zamoraa, M. E.; Rojas-Melgarejoa, F.; García-Canovasb, F.; García-Ruiz, P. A. J Biotechnol 2006, 126, 295.
12. Rebros, M.; Rosenberg, M.; Mlichova, Z.; Kristofíkova, L.; Paluch, M. Enzyme Microb Technol 2006, 39, 800.
13. Sari, M.; Akgöl, S.; Karatas, M.; Denizli, A. Ind Eng Chem Res 2006, 45, 3036.
14. Torres, R.; Pessela, B. C. C.; Mateo, C.; Ortiz, C.; Fuentes, M.; Guisan, J. M.; Fernandez-Lafuente, R. Biotechnol Prog 2004, 20, 1297.
15. Alonso-Morales, N.; Lopez-Gallego, F.; Betancor, L.; Hidalgo, A.; Mateo, C.; Fernandez-Lafuente, R.; Guisan, J. M. Biotechnol Prog 2004, 20, 533.
16. Gupta, M. N.; Jain, S.; Roy, I. Biotechnol Prog 2002, 18, 78.
17. Tishchenko, G.; Dybal, J.; Meszaroova, J. K.; Sedlakova, Z.; Bleha, M. J Chromatogr A 2002, 954, 115.
18. Denizli, F.; Denizli, A.; Arica, M. Y. Polym Int 1999, 48, 360.
19. Handa, T.; Hirose, A.; Yoshida, S.; Tsuchiya, H. Biotechnol Bioeng 1982, 24, 1639.
20. Turkmen, D.; Yavuz, H.; Denizli, A. Int J Biol Macromol 2006, 38, 126.
21. Uhlich, T.; Ulbricht, M.; Tomaschewski, G. Enzyme Microb Technol 1996, 19, 121.
22. Wang, F.; Guo, C.; Liu, H. Z.; Liu, C. Z. J Mol Catal B: Enzym 2007, 48, 1.
23. Lee, D. D.; Lee, G. K.; Reilly, P. J.; Lee, Y. Y. Biotechnol Bioeng 1980, 22, 1.
24. Denizli, A.; Say, R.; Garipcan, B.; Emir, S.; Karabakan, A.; Patir, S. Sep Purif Technol 2003, 30, 3.
25. Garipcan, B.; Denizli, A. Macromol Biosci 2002, 2, 135.
26. Andac, M.; Say, R.; Denizli, A. J Chromatogr B 2004, 811, 119.
27. Denizli, A. J Chromatogr B 2002, 772, 357.
28. Fowkes, F. M. J Adhes Sci Technol 1987, 1, 7.
29. Blanco, R.; Arai, A.; Grinberg, N.; Yarmush, D. M.; Karger, B. L. J Chromatogr 1989, 482, 1.
30. Case, D. A.; Cheatham, T. E.; Darden, T.; Gohlke, H.; Luo, R.; Merz, K. M., Jr.; Onufriev, A.; Simmerling, C.; Wang, B.; Woods, R. J Comput Chem 2005, 26, 1668.

31. Pang, Y. P. Proteins 2001, 45, 183.
32. Lindorff-Larsen, K.; Piana, S.; Palmo, K.; Maragakis, P.; Klepeis, J. L.; Dror, R. O.; Shaw, D. E. Proteins 2010, 78, 1950.
33. Miller, G. N. Anal Chem 1959, 81, 426.
34. Ko, Y. C.; Ratner, B. D.; Hoffman, A. S. J Colloid Interface Sci 1981, 82, 25.
35. Emir, S.; Say, R.; Yavuz, H.; Denizli, A. Biotechnol Prog 2004, 20, 223.
36. Akgöl, S.; Yavuz, H.; Senel, S.; Denizli, A. React Funct Polym 2003, 55, 45.
37. Sanjay, G.; Sugunan, S. Catal Commun 2005, 6, 525.
38. Yilmaz, F.; Yilmaz, E.; Uzun, L.; Denizli, A.; Hacettepe. J Biol Chem 2007, 35, 149.
39. Linden, A.; Mayans, O.; Meyer-Klaucke, W.; Antranikian, G.; Wilmanns, M. J Biol Chem 2003, 278, 9875.
40. Arica, Y.; Yavuz, H.; Patir, S.; Denizli, A. J Mol Catal B: Enzym 2000, 11, 127.